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PRINCIPAL INVESTIGATOR: Bo Xu, Ph.D.

CONTRACTING ORGANIZATION: Louisiana State University
New Orleans, LA 70112

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Bo Xu, Ph.D.

E-mail: bxu@lsuhsc.edu

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14. ABSTRACT

The focus of this project is to evaluate a newly developed small peptide on its ability to block DNA damage signaling pathways and to sensitize prostate tumor cells to radiotherapy. One of the critical DNA damage pathways which determine radiosensitivity is medicated by ATM and its phosphorylation of downstream targets, including Structural Maintenance of Chromosomal protein one (SMC1). Previously we have demonstrated that small fusion peptides containing SMC1 phosphorylation sequences can inhibit ATM activity. During the last performance period, we have characterized the inhibitory effect of the THM-SMC1 peptide on cellular response to radiation and found the peptide can abolish radiation induced S-phase checkpoint and decrease prostate tumor cell clonogenic survival. Current experiments are focusing on the mechanistic insight on how these inhibitory peptides work.

15. SUBJECT TERMS
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Introduction:

Critical controllers of cellular sensitivity to ionizing radiation (IR: used for radiotherapy) include the Ataxia-Telangiectasia-Mutated (ATM) protein kinase and its downstream target the Structural Maintenance of Chromosome protein one (SMC1)(Kim et al., 2002; Kitagawa et al., 2004; Yazdi et al., 2002). ATM phosphorylation of SMC1 at two serine sites is required for limiting the amount of radiation sensitivity. Our preliminary data have demonstrated that a small peptide containing ATM-mediated SMC1 phosphorylation sequence has an inhibitory effect on SMC1 phosphorylation and S-phase checkpoint activation after DNA damage. Therefore we hypothesized that a peptide containing this SMC1 short sequence, when linked with an tumor homing motif (THM), can be a specific inhibitor to the ATM-mediated signaling pathways and a powerful radiosensitizer for prostate tumor radiotherapy. This hypothesis has been tested by studying the effect of synthetic peptides that aim to block the in vivo phosphorylation events on prostate cancer cellular response to IR. We have generated three peptides, which include THM only (serving as a negative control), THM-wtSMC1 (as to-be-tested peptide), and THM-mtSMC1 (designed as a negative control with a possibility of inhibitory effects). In year one, our goals were to further characterize the fusion peptides.

Body:

A. Determine cellular toxicity of the peptides on normal prostate cells and prostate tumor cells. (SOW Task 1a)

Since our goal is to evaluate a potential radiosensitizer, the first thing we were to test is the cytotoxicity of the peptides to normal and tumoral prostate cells. To achieve this goal, we utilized normal prostate epithelial cells (provided by Dr. Shahriar Koochekpour), PC-3, DU-145 and LNCap cells. ID_{50} of the peptides was determined by cell viability assays, which were measured by the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Both dose response and time course of exposure to the peptides were studied. As shown in Figure 1, the three peptides, when doses are lower than 50 μM for 2-hour exposure, showed minimal effects on cell survival. Meanwhile, when doses are higher than 50 μM, all three peptides have moderate toxicity to the cells. It is noted that a positive control (Taxol) was utilized in these experiments. In the time course experiments (Figure 2), different time exposure, with up to 24 hours and at a dose of 10μM, posed minimal toxicity to the cells. The effect of the peptides on cell cycle progression was also investigated. Cells treated with peptides were harvested, fixed in 70% of ethanol, and stained with propidium iodide, followed by flow cytometric analysis. No change of basal cell cycle distribution is detected (data not shown). These observations suggest that the THM-fusion peptides have minimal cytotoxicity to

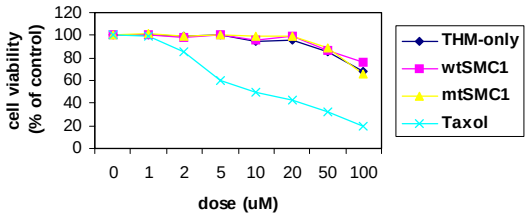

Figure 1. The THM-SMC1 fusion peptides possess no cytotoxicity at doses lower than 50 μM. PC-3 cells were treated with indicated concentrations of the peptides or Taxol for 2 hrs. Cellular viability was assessed by the MTT assay.

normal prostate cells and prostate tumor cells; therefore they can be tested as pure radiosensitizers.

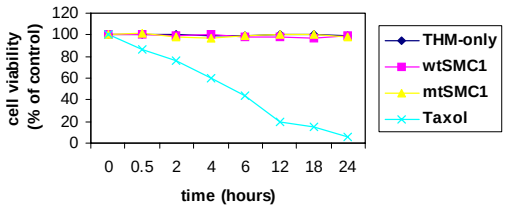

Figure 2. The THM peptides have limited cytotoxicity (time course). Cells were treated with 10 μM of the peptides or Taxol for indicated time. The viability was assessed by the MTT assay.

B. Investigate the effect of the THM-SMC1 peptide on ATM activation and SMC1 phosphorylation after IR. (SOW Task 1b)

Our hypothesis is that the wt SMC1 peptide can target the ATM-SMC1 pathways. To test this hypothesis, we investigated the effect of these peptides on ATM activation and SMC1 phosphorylation in response to IR. Western blot analyses were performed to assess ATM autophosphorylation (using a commercially available antibody recognizing phosphorylation of Serine 1981 from Rockland) and SMC1 phosphorylation (using a commercially available antibody recognizing phosphorylation of Serine 966 from Bethyl Laboratories Inc.) after IR in PC-3 cells treated with the peptides. Total amount of ATM and SMC1 were immuno-blotted and used as loading controls. As shown in Figure 3, while the THM only peptide does not change IR-induced ATM activation and SMC1 phosphorylation, the wtSMC1 peptide significantly diminished ATM autophosphorylation and SMC1 phosphorylation. It is interesting to observe that the mtSMC1 peptide functioned similar to THM-only with not noticeable effect on ATM and SMC1 phosphorylation. This is consistent with our previous observation that the mtSMC1 has minimal effects on activation of the S-phase checkpoint after IR.

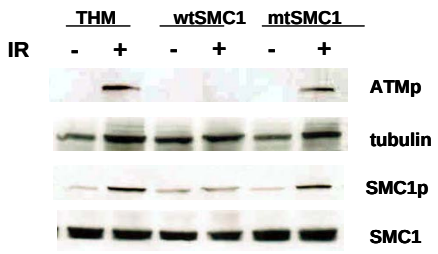

Figure 3. The THM-wtSMC1 peptide abolished ATM activation and SMC1 phosphorylation after IR. PC-3 cells were treated with indicated peptides, followed by 6 Gy of IR. Whole cell lysates were harvested and immunoblotted with indicated antibodies.

C. Investigate the effect of the THM-SMC1 peptide on IR-induced G2/M checkpoint. (SOW Task 1c)

One of the endpoints to evaluate the THM peptides is to study the effect on the G2/M checkpoint. The G2/M checkpoint is dependent on the ATM and BRCA1 proteins. ATM phosphorylation of BRCA1 regulates the checkpoint, whereas ATM phosphorylation of SMC1 is independent of the checkpoint (Xu et al., 2002; Kim et al., 2002). If the THM-wtSMC1 peptide only interferes with the ATM-SMC1 signal, they should have minimal

effect on activation of the G2/M checkpoint. However, if the THM-wtSMC1peptide target ATM kinase activity, then the G2/M checkpoint should be abrogated by the peptide. We previously looked at the G2/M checkpoint in PC-3 cells and concluded a normal response to IR in the cells. When treated with the THM only peptide, the cells maintain an intact G2/M checkpoint as measured by histone H3 phosphorylation, an event required for chromosome condensation and mitotic entry. However, the THM-wtSMC1 peptide partially abrogates the checkpoint (Figure 4). Similar to its effect on the S checkpoint, the THM-mtSmc1 peptide has less effect on the process.

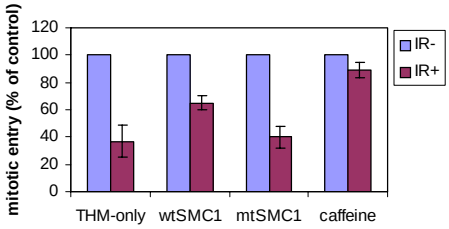

Figure 4. The THM-wtSMC1 peptide can partially abrogate the G2/M checkpoint. PC-3 cells treated with the peptides were radiated with 0 or 6 Gy of IR. The G2/M checkpoint was assessed by histone H3 staining followed by flow cytometry analysis. Error bars represent averages of at least three samples.

D. Investigate the effect of the THM-SMC1 peptide on radiation sensitivity. (SOW Task 1d)

We then tested radiation sensitivity in PC-3 cells. Colony formation assays in soft agar were performed in six-well plates using $1X10^4$ cells per well. The bottom layer contains 2 ml of 0.6% Noble agar (Difco, Becton Dickinson), penicillin (100U/ml), streptomycin (100µg/ml), 10% FBS in DMEM medium. The top layer contains 1 ml of 0.3% Nobel agar, penicillin (100Ug/ml), streptomycin (100µg/ml), 10% of FBS, DMEM and 10,000 cells. Plates were treated with the peptides and then be radiated at 0-6Gys. After 2-week incubation, the numbers of colonies (those with more than 50 cells will be counted as one surviving colony) were counted and the surviving fractions were calculated. Shown in Figure 5 is the surviving fraction at 2Gy of IR (SF2). The THM-only peptide and the mtSMC1 peptide did not change SF2, while THM-wtSMC1 significantly decreases the SF2. More colony formation experiments are underway and some critical parameters of radiation sensitivity will be obtained.

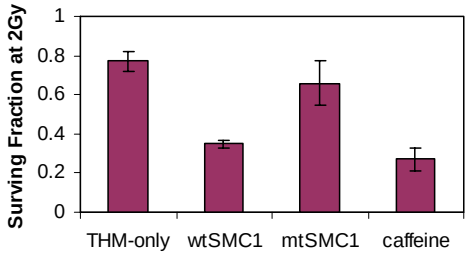

Figure 5. The THM-wtSMC1 peptide increases PC-3 cell radiosensitivity. PC-3 cells were treated with indicated peptides followed by mock or up to 6 Gy of IR. Colony formation assay was conducted and the surviving fraction was calculated. Shown are surviving fractions at 2Gy (SF2). Error bars represent averages of at least three samples.

Key research accomplishments:

We have successfully finished the proposed experiment in aim 1.

1. We have demonstrated that the THM-wt SMC1 peptide that contains the wild-type SMC1 phosphorylation sequence has an inhibitory effect on ATM activation and subsequent SMC1 phosphorylation after IR;
2. This peptide does not possess cellular toxicity to normal or tumoral prostate cells; and
3. It can increase radiation sensitivity of prostate tumor cells.

Reportable outcomes:

We will present our observations in major scientific meetings, such as the 2006 Annual Meeting of American Association of Cancer Research. An abstract has been submitted to the meeting.

Conclusions

The THM-fusion SMC1 peptides have minimal toxicity to normal and prostate tumor cells, clearing a way for testing the peptide as a pure radiosensitizer. The THM-wt SMC1 can inhibit ATM activation, SMC1 phosphorylation, and the G2/M checkpoint. It can also increase prostate tumor cell radiosensitivity.

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Appendices

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